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Validation of Reference Genes for Transcriptional Analyses in *Pleurotus ostreatus* by Using Reverse Transcription-Quantitative PCR

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Recently, the lignin-degrading basidiomycete *Pleurotus ostreatus* has become a widely used model organism for fungal genomic and transcriptomic analyses. The increasing interest in this species has led to an increasing number of studies analyzing the transcriptional regulation of multigene families that encode extracellular enzymes. Reverse transcription (RT) followed by real-time PCR is the most suitable technique for analyzing the expression of gene sets under multiple culture conditions. In this work, we tested the suitability of 13 candidate genes for their use as reference genes in *P. ostreatus* time course cultures for enzyme production. We applied three different statistical algorithms and obtained a combination of stable reference genes for optimal normalization of RT-quantitative PCR assays. This reference index can be used for future transcriptomic analyses and validation of transcriptome sequencing or microarray data. Moreover, we analyzed the expression patterns of a laccase and a manganese peroxidase (*lacc10* and *mnp3*, respectively) in lignocellulose and glucose-based media using submerged, semisolid, and solid-state fermentation. By testing different normalization strategies, we demonstrate that the use of nonvalidated reference genes as internal controls leads to biased results and misinterpretations of the biological responses underlying expression changes.

The basidiomycete *Pleurotus ostreatus* is an efficient producer of extracellular enzymes, such as laccases (Lacs; EC 1.10.3.2) and manganese peroxidases (MnPs; EC 1.11.1.13), which are notable for their application in multiple industrial and biotechnological processes (1, 2). It has a special relevance in agroindustry due to its worldwide cultivation for mushroom production, and it has also been studied for its nutritional and medicinal value (3). Currently, two genomic sequences of *P. ostreatus* are publicly available, and it has become a very popular model organism for the study of fungal genetics and comparative genomics (4–6). Additionally, many studies on the production of ligninolytic enzymes, with a focus on culture optimization for enzyme production, have recently been published. Most of these enzymes are encoded by genes organized in gene families, which are occasionally arranged into clusters as a result of gene duplications.

Despite the information uncovered by sequencing efforts, substantial work on the functional characterization of gene family members remains to be performed. In this sense, transcriptomic analyses are powerful tools for providing an understanding of gene regulation and the presumptive function of genes. Despite the high productivity and low cost of transcriptome sequencing (RNA-seq), reverse transcription (RT) followed by real-time PCR (RT-quantitative PCR [RT-qPCR]) is likely the best option for analyzing the expression patterns of certain genes under multiple conditions. Additionally, it is the most reliable technique for validating RNA-seq and microarray data because of its specificity, reproducibility, and capacity for detecting and measuring tiny amounts of nucleic acids in a wide quantification range (7).

Since its first appearance in 1993 (8), RT-qPCR has evolved into a well-established technology. Many studies of new quantification models (9), PCR efficiency calculations (10, 11), or baseline determinations (12) have been performed to improve the accuracy of RT-qPCR. Several types of software that implement these approaches are currently available (13, 14). Within the two possible quantification strategies (relative and absolute), relative quantification is a very common strategy for analyzing the expression levels of a target gene under multiple conditions or in multiple samples. In this method of quantification, the mRNA levels of the target gene are compared to the level of expression of a reference gene that passes through all the steps along with the target gene. The use of an internal standard is essential to compensate for the variance introduced in every step of the process, such as RNA quantification, reverse transcription, or pipetting (9, 15–17). The ideal reference gene should have constant expression, regardless of the sample, condition, cell, or treatment used (16, 18). Housekeeping genes, such as 18S rRNA, β-actin, β-tubulin, or glyceraldehyde-3-phosphate dehydrogenase, have commonly been used as reference genes. Unfortunately, an overwhelming number of studies have shown that such genes are often unacceptable for RT-qPCR normalization due to unstable expression (19, 20). Additionally, there is strong evidence that the use of more than one reference gene is more reliable than the use of only one (7, 16, 18). The choice of adequate internal standards is crucial to obtain a successful and unbiased determination of expression, which is essential to analyze the expression stability of reference genes in validation assays.

Several methods of analysis of reference gene panels have been proposed (16, 18, 20–22), and studies using these methodologies

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with different species are becoming more frequent. In this work, we tested the suitability of 13 candidate genes for their use as reference genes in *P. ostreatus* using different culture conditions and growing stages. We applied three different statistical algorithms and obtained a combination of reference genes that can be used for future *P. ostreatus* transcriptomic studies. Moreover, we analyzed the relative expression of laccase and manganese peroxidase genes (*lacc10* and *mnp3*, respectively) in lignocellulose and glucose-based media using different normalization strategies. Our results demonstrate that the use of unstably expressed genes as internal controls leads to biased data and misinterpretations of the biological responses underlying expression changes.

MATERIALS AND METHODS

Fungal strain. In this study, dikaryotic strain N001 of *Pleurotus ostreatus* var. florida was used. The genomic sequences of its monokaryotic protoclones, PC15 and PC9 (23), were obtained from the JGI genome portal (http://genome.jgi.doe.gov/).

Culture media and growing conditions. Mycelia were grown on petri dishes with solid PDA medium (Scharlab, S.L., Barcelona, Spain) and maintained in the darkness at 24°C. Inocula were prepared in Erlenmeyer flasks containing 150 ml of SMY medium (10 g of sucrose, 10 g of malt extract, 4 g of yeast extract per liter) and 4 mycelium pieces of approximately 0.25 cm^2. These cultures were maintained in the darkness at 24°C for 6 days with orbital shaking (125 rpm) and homogenized in an Omnimixer. Fifteen milliliters of the homogenized inoculum was added to the final media. Regarding the experimental conditions, three media were used: (i) submerged fermentation (SmF) cultures set on Erlenmeyer flasks with 135 ml of SMY medium; (ii) semi-solid-state fermentation (s-SSF) cultures containing 10 g of milled wheat straw and 70 ml of distilled water set in glass flasks with a surface-to-volume ratio of 1 cm^{-1}, and (iii) solid-state fermentation (SSF) cultures in polypropylene plastic bags containing 15 g of poplar chips of approximately 200 by 50 mm and 22.5 ml of distilled water. All cultures were inoculated with 15 ml of inoculum and incubated in the darkness at 24°C. SmF cultures were kept on an orbital shaker (125 rpm), whereas s-SSF and SSF cultures were incubated under static conditions.

Experimental conditions. To include different growth stages in our analyses, the sampling times were based on the findings of previous studies analyzing the variations observed during *P. ostreatus* growth (24). Three independent biological replicates were sampled for each culture medium. In this sense, we sampled SmF cultures on days 1, 3, 7, 11, and 15; s-SSF cultures on days 3, 7, 11, 15, and 30; and SSF cultures on days 3, 7, 11, 15, and 30.

Nucleic acid extraction and real-time qPCR. Mycelia were harvested, frozen, and ground in a sterile mortar with liquid nitrogen. Total RNA was extracted from 200 mg of deep-frozen tissue using a fungal RNA E.Z.N.A. kit (Omega Bio-Tek, Norcross, GA, USA); its integrity was estimated by denaturing electrophoresis on 1% (wt/vol) agarose gels. Nucleic acid concentrations were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and the purity of the total RNA was estimated by determination of the 260-nm/280-nm absorbance ratio. Samples were treated with DNase, using 1 U of RQ1 DNase (Promega, Madison, WI, USA) per μg of RNA. Subsequently, the samples were purified using an RNeasy MinElute cleanup kit (Qiagen Iberia S.L., Madrid, Spain), and the final RNA concentration was set to 400 ng/μl. Total RNA (800 ng per sample) was reverse transcribed into cDNA in a 20-μl volume using an iScript cDNA synthesis kit (Bio-Rad, Alcobendas, Spain). RT was carried out in a thermal cycler (MJ Research, Inc.), and the protocol consisted of 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C.

RT-qPCRs were performed in a CFX96 real-time system (Bio-Rad Laboratories, S.A.) using SYBR green dye to detect product amplification. The primers shown in Table 1 were used for the amplification of the transcripts from the reference genes and the *lacc10* and *mnp3* genes. Each reaction mixture was set to a final volume of 20 μl containing 10 μl iQ SYBR green supermix (Bio-Rad Laboratories, S.A.), 2 μl of 3 μM stock forward and reverse primers (Table 1), 1 μl of a 1:20 dilution of the RT product, and 5 μl of sterile water. The amplification program consisted of 5 min at 95°C and 40 cycles of 15 s at 95°C, 30 s at 63°C, and 15 s at 72°C, followed by a final melting curve analysis in which the temperature was increased at increments of 0.5°C every 5 s in a linear gradient from 65 to 95°C. The specificity of each reaction was confirmed by inspection of the melting curve profiles. Each reaction was performed in triplicate, and nontemplate controls (NTCs) were included for each primer set. An experimentally validated interplate calibrator (IPC) was used to compensate for interplate variation. Crossing-point (Cp) values and relative fluorescence units were recorded, and the latter were used to calculate amplification efficiencies by linear regression using the LinReg program (25).

Reference gene panel. We selected 13 genes of different functional classes as candidates (Table 2). Our gene panel contained (i) housekeeping genes, such as tubulin (*tub*), actin (*actin1*), or cytochrome *c* (*cyt-c*); (ii) genes chosen on the basis of stable expression in several RNA-seq and qPCR experiments, such as lipase (*lip*) or methylthioadenosine phosphorylase (*phos*) (26, 27); and (iii) the *sar1* gene, described to be the ideal reference gene in the ascomycetes *Aspergillus niger* (28) and *Trichoderma reesei* (29). The 13 candidates were classified according to their suitability as internal standards using the geNorm (18), NormFinder (16), and BestKeeper (21) algorithms.

Data normalization and quantification strategy. Data preprocessing was carried out using GenEx software (MultiD Analyses) and included interplate calibration, efficiency correction, and normalization using different sets of reference genes and the calculation of relative quantities (RQs in equation 1) as well as the relative expression ratios (expression in equation 2). Samples harvested from SmF cultures at day 1 were used as control samples in the latter quantification.

$$\mathrm{RQ} = 2^{-\Delta \mathrm{Cp}_E} \quad (1)$$

$$\text{Expression} = 2^{-(\Delta\Delta \mathrm{Cp}_E)} \quad (2)$$

where Cp_E is the efficiency-corrected crossing point. $\Delta\mathrm{Cp}_E$ in equation 1 represents the difference between the Cp_E of the target gene and the mean Cp_E for the reference genes. The expression value in equation 2 represents expression determined by the classical $\Delta\Delta C_T$ threshold cycle (C_T) method (15).

To analyze the impact of reference gene selection on the relative expression calculations, we obtained the expression of the laccase *lacc10* gene and the manganese peroxidase *mnp3* gene using different normalization strategies: (i) the mean expression of 12 genes, (ii) the mean expression of the top 3 reference genes from the ranking, (iii) the expression of the most stable reference gene, (iv) the expression of the least stable reference gene, and (v) the amount of total RNA (no reference genes).

Statistical analyses. Heat map and hierarchical clustering analyses were performed on autoscaled expression data using Wards' algorithm and the Euclidean distance. A Mann-Whitney test was used to uncover significant differences in the relative expression ratios calculated using the different normalization strategies. All statistical analyses were performed using GenEx.

RESULTS AND DISCUSSION

The expression levels of the 13 genes were measured under each of the 15 experimental conditions (see Materials and Methods). The mean crossing-point (Cp) values ranged from 18 to 25 cycles (Fig. 1). The standard deviation (SD) among the 15 different conditions was clearly variable, ranging from 0.64 cycle for *vsn* to 1.66 cycles for *lip*. The gene *chs* showed the lowest level of expression (highest Cp value, 28.04 cycles), whereas *actin1* showed the highest level of expression (lowest Cp value, 16.71 cycles). Other housekeeping genes, such as *tub*, *cyt*, or *gapdh1*, also showed high

TABLE 1 Identifiers, product lengths, and amplification efficiencies of the primers used in this study

Gene	Transcript identifier		Orientation[a]	Sequence	Amplicon length (bp)	Amplification efficiency (%)	Reference or source
	PC15 (v2.0)	PC9 (v1.0)					
pep	1092697	115017	Fw	TGATTCCAGAGGACAAGGACGCAA	148	89	26
			Rv	AAATCTTCCGCGATACGGGTCACT			
cyc	1035989	86026	Fw	ACCTGGCGTCGTTATCTCAAGTGT	122	90	26
			Rv	TTGGCCGAACAGCGAATGGTTTAC			
phos	49987	49690	Fw	CATCGCAAATCATCGATCGCACCA	125	89	26
			Rv	GCTCTCCAGCCAATGCACCAATTT			
tub	1091050	117235	Fw	AGGCTTTCTTGCATTGGTACACGC	136	85	27
			Rv	TATTCGCCTTCTTCCTCATCGGCA			
lip	1052421	75430	Fw	AGGTTTGGCGGGATACAATACGGA	132	83	26
			Rv	ATGCAAGCATCATTTGCGCCGAAC			
vsn	1111692	115919	Fw	AGGTGGAAGGACGTCGAAATGGAA	146	85	This study
			Rv	AAGACGTCTCTGTGCCGCTGTATT			
cyph	1058252	72928	Fw	GACATTGCTATCGACTCCCAG	84	88	39
			Rv	GAAATTCCTTGCAGTCTTGGG			
actin1	1087906	114148	Fw	AGTCGGTGCCTTGGTTAT	129	90	27
			Rv	ATACCGACCATCACACCT			
cyt	1113744	112752	Fw	GCCTCATAAAGTCGGTCCTAAC	127	87	26
			Rv	CTCAAATAGGGTGTTCTCGTCC			
gapdh1	1090672	88005	Fw	TGGTCCATCGCATAAGGA	194	88	27
			Rv	ACACGGAAGGACAAACCA			
pas	1057954	87317	Fw	CATCCAGACGAATTTCCTAGAG	169	87	This study
			Rv	GAGACACTTCCAACGAGAAC			
sar1	1052294	69194	Fw	GGATAGTCTTCCTCGTCGATAG	133	87	This study
			Rv	GGGTGCGTCAATCTTGTTAC			
chs	28823	51952	Fw	CGAAGGACTATTATGCGAGTG	170	87	This study
			Rv	TGAATGCCAGTACGAAGATAAG			
lacc10	1089723	81117	Fw	CCTACTTCCCCTTTGGCTATC	122	85	39
			Rv	ATGACGAGCAAAGAGTGACC			
mnp3	1089546	51690	Fw	ATGGACAAGTTGGCTACACTCGGT	139	87	27
			Rv	CACACGCTTGCTCGATGTTGTTCA			

[a] Fw, forward; Rv, reverse.

levels of transcription, in concordance with the important roles that they play in most cellular processes.

Stability of reference genes: NormFinder, geNorm, and BestKeeper analyses. The NormFinder algorithm identified *sar1* to be the most stable reference gene because its NormFinder standard deviation (SD_N) was lower than the SD_Ns of the other candidates (0.27 cycle). Interestingly, the gene showing lower intersample variation in the exploratory analysis of Cp values (the *vsn* gene; Fig. 1) was ranked 10th, evidence that raw Cp intersample variation itself is not a reliable indicator of expression stability.

The accumulated SD_N (Acc. SD_N) was explored to determine the optimal number of reference genes to be used. Our results showed that a reference index composed of 12 genes (*sar1*, *phos*, *pep*, *lip*, *cyph*, *cyt*, *gapdh1*, *actin1*, *chs*, *vsn*, *pas*, and *tub*) would be the most appropriate for data normalization according to its low Acc. SD_N (0.23 cycle) (Fig. 2B). Nevertheless, the difference between this index and another composed of the three best reference genes (*sar1*, *phos*, *pep*) was only 0.03 cycle. Thus, the use of three reference genes for normalization (Acc. SD_N, 0.26 cycle) is reasonable and avoids the increased cost and the loss of efficiency of using 12 genes.

To obtain more robust results, the data were again analyzed by taking into account the intra- and intergroup variations in gene expression among the three culture media (see Tables S1 and S2 in the supplemental material). Genes showing high intergroup variation (cutoff SD_N, 0.65 cycles) were discarded because the inclusion of unstable genes in the analysis would lead to a less reliable ranking of expression stability. After removing the data for *tub*,

TABLE 2 Description of reference genes used in this study

Gene name	Chromosome localization	Function
pep	IV	Peptidase activity
cyc	III	Union protein
phos	IV	Methylthioadenosine phosphorylase activity
tub	XI	Structural activity
lip	I	Lipase
vsn	III	Intracellular transport
cyph	VII	Isomerase activity
actin1	I	Structural activity
cyt	VIII	Electron transport
gapdh1	VII	Glycolysis
pas	VII	Signal transduction activity
sar1	I	GTP binding
chs	V	Chitin biosynthetic process

vsn, *pas*, and *actin1*, NormFinder was run again, considering that all of the samples formed one group (Fig. 2C). The results showed a profile very similar to that found in the first run. The gene *sar1* was again the best and was classified with an SD_N of 0.29 cycle, followed by *phos* and *pep*; *cyc* was the least stable. Nevertheless, the analysis of the Acc. SD_N showed that in this instance, the ideal reference index was composed of three genes displaying an Acc. SD_N of 0.24 cycle (Fig. 2D). This deviation is useful for evaluating the systematic error introduced when normalizing RT-PCR data. In our case, this value falls in the range of variability that is often found between technical replicates. Thus, we can conclude that the systematic error (Acc. SD_N) introduced by our proposed reference index (the average for *sar1*, *phos*, and *pep*) is admissible for an accurate expression analysis.

To give more strength to the results, we analyzed the same data set with the geNorm and BestKeeper programs. The geNorm tool identified the combination of *sar1* and *pep* to be the most stable gene pair (expression stability value [*M*], 0.62), and *cyc* was again identified to be the least stable candidate (Fig. 3). Because BestKeeper can analyze up to 10 genes, we chose the top 10 candidates from the NormFinder classification as input data (80% of them were also within the top 10 in the geNorm ranking). According to the descriptive statistics released by BestKeeper, *sar1* and *phos* displayed the lowest coefficient of variance (3.97% and 3.67%, respectively) and standard deviation (0.80 cycle and 0.87 cycle, respectively) (Table 3). Following other authors' considerations (21), the results for genes showing a standard deviation of greater than 1 cycle were considered inconsistent and removed from the analysis; the remainder were used in further analyses. The BestKeeper algorithm calculates the pairwise correlations between Cp values and the BestKeeper index (BI) on the basis of the geometric mean for stable genes (SD, <1). Thus, genes showing a higher correlation coefficient (*r*) and a significant *P* value ($P < 0.05$) were considered the most stable. According to this, *sar1* was the first to be classified in the ranking ($r = 0.94$, $P < 0.05$). The high correlation between this gene and the BI undermines its stable expression and, thus, its suitability as a reference gene.

Reference gene stability ranking. According to the four methodologies used, *sar1* was the most stable candidate gene (Table 4). The reference gene *pep* appeared at the top three positions in all the rankings, similar to *phos*, with the exception of those made by geNorm, where it occupied the 5th place. When the top six reference genes identified by NormFinder were compared with those identified by the other methodologies, five of the six genes were the same in the BestKeeper results, whereas four of the six were the same in the geNorm results. Small discrepancies between the three algorithms are common (30–33); nevertheless, results are usually consistent within the best-classified genes, as was found in this study.

Interestingly, several housekeeping genes, such as *actin1*, *gapdh1*, or *tub*, were among the less stable reference genes tested. These genes have been widely used as internal standards for the normalization of RT-PCR and RT-qPCR data. During the last decade, many studies have revealed the instability of housekeep-

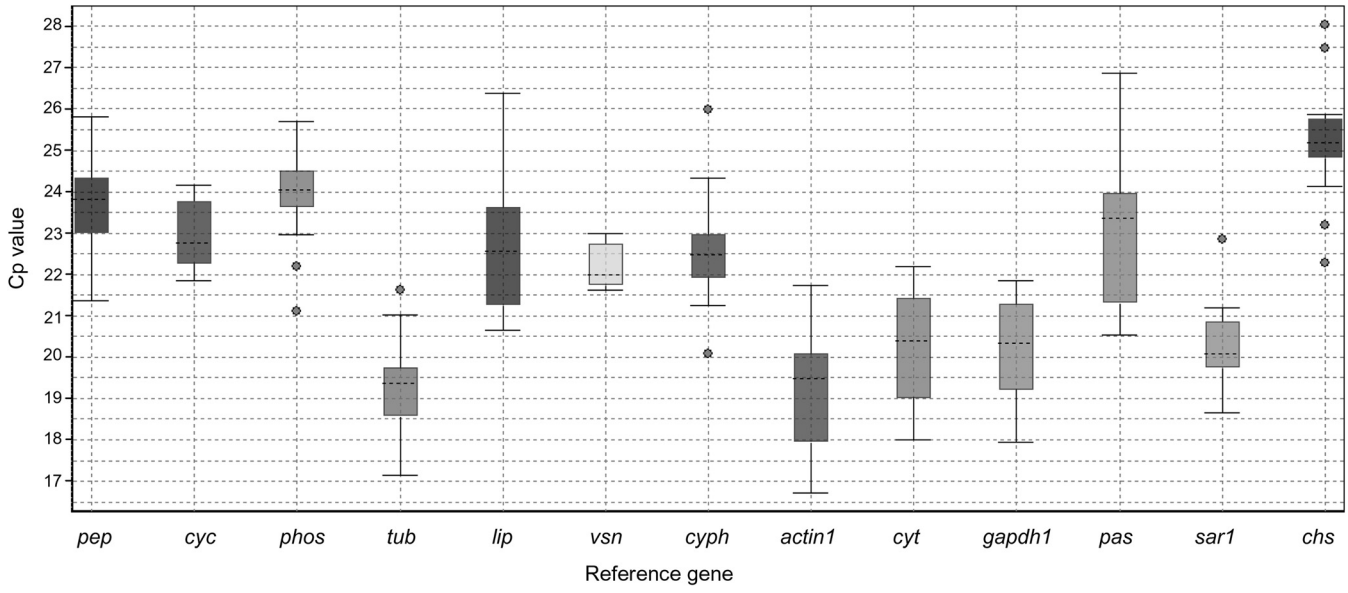

FIG 1 Variability of Cp values of 13 reference genes tested under the 15 experimental conditions.

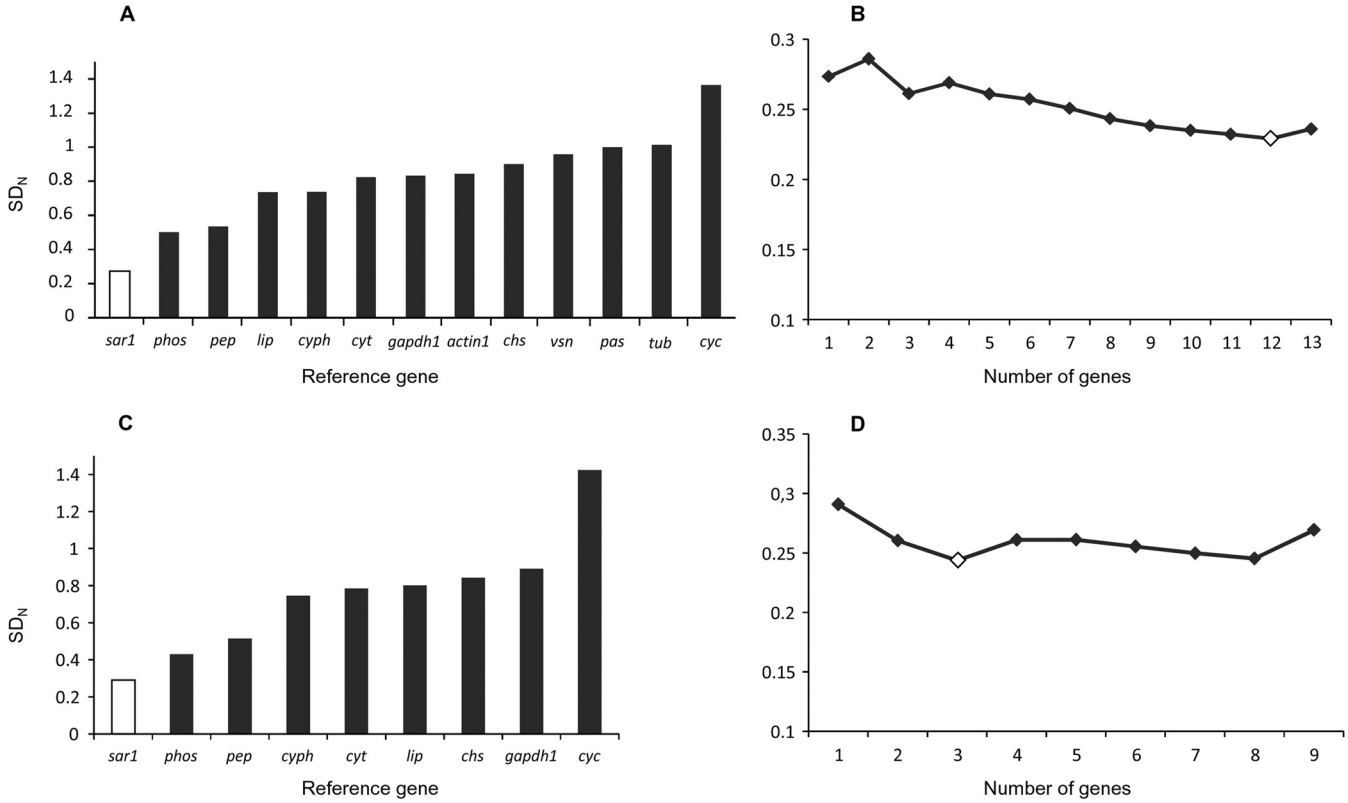

FIG 2 Summary of NormFinder analysis of the candidate genes by use of 13 genes (A and B) and 9 genes (C and D). The accumulated SD_N (Acc. SD_N) values of increasing numbers of reference genes are shown in panels B and D.

ing gene expression (31, 34, 35), although they can also be acceptable internal standards under certain conditions. This fact is evidence that gene expression stability varies with the gene, species, tissue, and culture conditions used (36, 37).

In summary, our results reinforce the fact that reference genes should never be chosen on the unique basis of suitability in other organisms. Therefore, if no data for a given species are available, experimental validation should always be the first step in RT-qPCR expression studies. In this sense, geNorm, NormFinder, and BestKeeper are the three methods commonly used for analyzing the stability of reference gene panels. Other statistical methods have been developed with the same aim (20, 22, 38) but are used less frequently, most likely due to the greater complexity of their application. BestKeeper has a limitation in the number of genes that can be analyzed. This is inconvenient when attempting to analyze larger (and, thus, more robust) reference gene panels. The geNorm tool calculates the best pair of genes on the basis of the variation with the other genes tested. In our case, its results were the most discordant. In general, the three methods are susceptible to producing the wrong results when introducing coregulated genes.

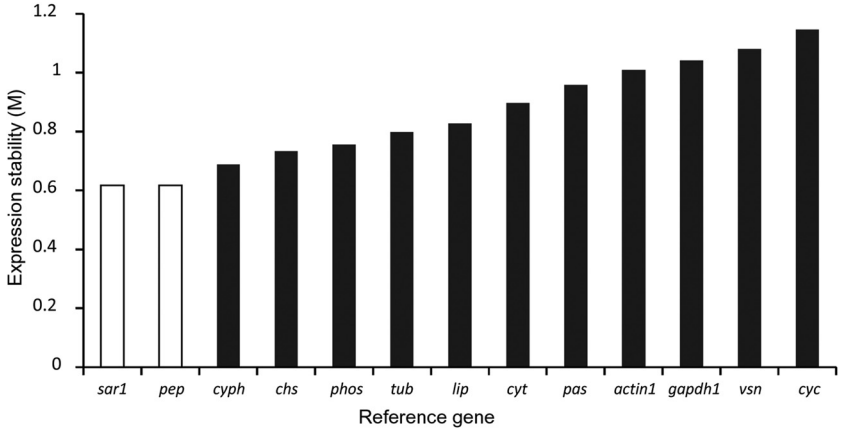

FIG 3 Summary of geNorm analysis of the candidate genes.

TABLE 3 Summary of BestKeeper results[a]

Gene	Cp value						
	Geometric mean	Arithmetic mean	Minimum	Maximum	SD	% CV	*r* value
pep	23.59	23.63	21.06	26.44	1	4.22	0.90
phos	23.78	23.81	20.7	26.02	0.87	3.67	0.89
lip	22.66	22.71	20.2	26.46	1.4	6.17	0.92
vsn	22.58	22.64	20.2	26.46	1.38	6.09	0.96
cyph	22.47	22.51	19.57	26.46	0.98	4.36	0.88
actin1	18.97	19.03	16.4	23.26	1.33	6.99	0.84
cyt	20.08	20.11	17.89	22.71	0.99	4.9	0.81
gapdh1	20.09	20.13	17.45	22.68	1.08	5.37	0.77
sar1	20.17	20.2	18.08	23.31	0.8	3.97	0.94
chs	25.06	25.1	21.57	28.69	1.08	4.28	0.84

[a] Data are for 45 repetitions of the analysis, and *P* was 0.001 for comparisons of all genes. CV, coefficient of variation.

The choice of the most appropriate tool is a difficult task, and due to the lack of a perfect standard for expression analyses, the use of several algorithms represents a reasonable way to proceed. Normalization of RT-qPCR data on the basis of data for multiple reference genes allows an evaluation of gene expression more precise than that obtained by use of a correction for a single reference gene (16, 18, 21). In this sense, the balance between costs and benefits plays an important role in the number of genes selected for normalization. The analysis of the optimal number of reference genes to be used revealed that the difference in Acc. SD_N values is not very large when the value obtained with three genes versus the value obtained with one gene are compared (0.24 versus 0.29 cycle; Fig. 2D). Nevertheless, the difference between using one gene and using three genes can be decisive in case of pipetting errors when the reference gene reaction is set up.

Taking into account the results obtained using the three algorithms, a reference index formed by *sar1*, *pep*, and *phos* could be considered the ideal internal standard for normalizing the RT-qPCR data for *P. ostreatus* species. Interestingly, the best reference gene detected in our study was *sar1*, an orthologue of the *sar1* GTPase of *Trichoderma reesei*, a species in which it was described to be an ideal reference gene (29). According to the conserved domains of this protein, *sar1* participates in important biological processes, such as GTP binding, intracellular transport, protein secretion, and signal transduction. Thus, it seems reasonable for the organism to maintain its constitutive transcription. This gene has also been validated to be stable in the ascomycete *Aspergillus niger* (28). According to its exceptional intragroup stability (SD_N, <0.1 cycle in every medium; see Table S1 in the supplemental material), its use as a single reference gene could be justified (although not recommended) when comparing the expression of time course samples from the same culture medium.

In order to extend our results to other fungal species, we performed an exploratory analysis using publicly available microarray data (see the Materials and Methods and Fig. S1 in the supplemental material). The level of expression of *sar1*, *pep*, and *phos* compared to the mean level of expression of all genes evidenced the apparent expression stability of our candidates (especially of *sar1*) in the fungal models *Phanerochaete chrysosporium*, *Coprinopsis cinerea*, and *Postia placenta*. These results should be taken with caution; they are not a direct validation of their general suitability for normalization of RT-qPCR data, as the exploratory approach used in these fungal models lacks sufficient statistical robustness (i.e., sufficient samples from different media or developmental stages are lacking). Nevertheless, according to the results obtained, we propose that the genes *pep*, *phos*, and, especially, *sar1* are good candidates to be included in RT-qPCR reference gene panels to be further validated with these species.

Impact of normalization strategy on gene expression quantification. To uncover the impact of the selection of reference genes on gene expression calculations, we analyzed the expression of *lacc10* and *mnp3* in all of the samples using five normalization strategies (Table 5). We obtained time course expression ratios (equation 2) for use as a reference for mycelium cultured on SmF medium harvested at day 1. The results showed clear evidence that

TABLE 4 Summary of stability rankings obtained by NormFinder, geNorm, and BestKeeper

Classification	Stability ranking[a]			
	NormFinder			
	13 genes	9 genes	geNorm	BestKeeper
1	***sar1***	***sar1***	***sar1***	***sar1***
2	***phos***	***phos***	***pep***	***pep***
3	***pep***	***pep***	*cyph*	***phos***
4	*lip*	*cyph*	*chs*	*cyph*
5	*cyph*	*cyt*	***phos***	*cyt*
6	*cyt*	*lip*	*tub*	*vsn*
7	*gapdh1*	*chs*	*lip*	*lip*
8	*actin1*	*gapdh1*	*cyt*	*chs*
9	*chs*	*cyc*	*pas*	*actin1*
10	*vsn*		*actin1*	*gapdh1*
11	*pas*		*gapdh1*	
12	*tub*		*vsn*	
13	*cyc*		*cyc*	

[a] Boldface indicates the stable reference genes for optimal normalization of RT-qPCR assays.

TABLE 5 Normalization strategies used in this analysis

Strategy	Gene(s)
I	*sar1*, *phos*, *pep*, *lip*, *cyph*, *cyt*, *gapdh1*, *actin1*, *chs*, *vsn*, *pas*, *tub*
II	*sar1*, *phos*, *pep*
III	*sar1*
IV	*cyc*
V	No reference gene

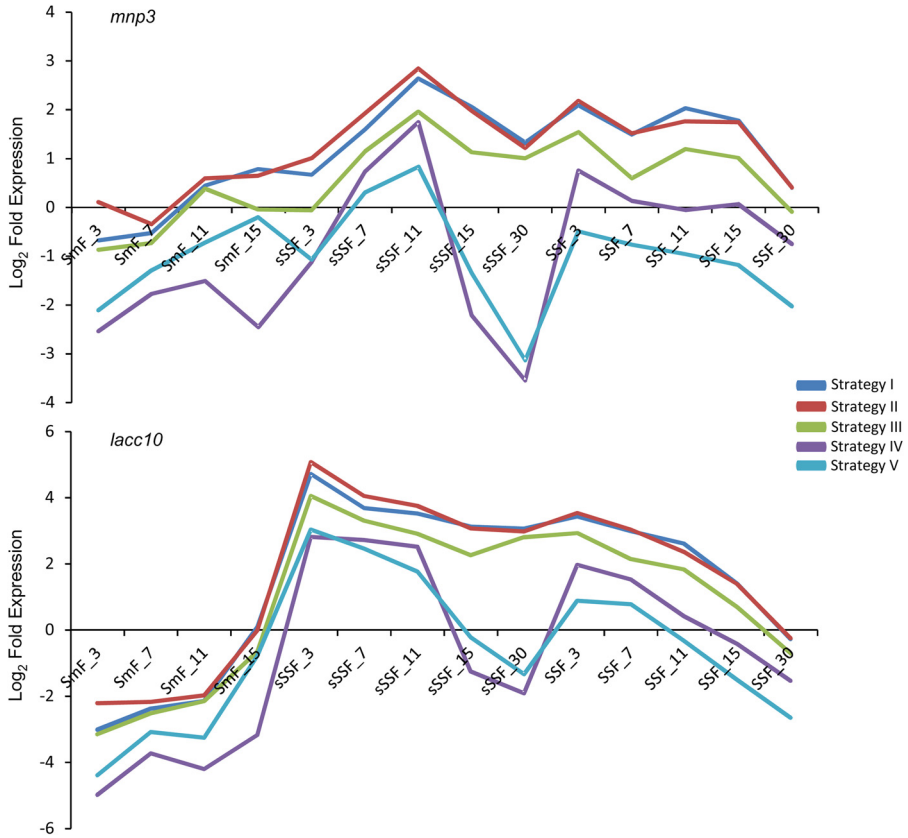

FIG 4 Time course gene expression trends (log_2) for *mnp3* and *lacc10* in SmF, s-SSF, and SSF media, obtained using five different normalization strategies.

the normalization strategy strongly influenced the expression results. In fact, in some cases, the expression ratios showed totally opposite patterns when they were normalized with validated versus nonvalidated reference genes. This was particularly evident in the expression of both genes in s-SSF and SSF media (Fig. 4 and 5). For example, at day 15 of s-SSF culture, *lacc10* showed an overexpression of 3.1 log_2-fold when using strategy II (the optimal reference index); nevertheless, the use of a single gene, *cyc* (strategy IV), yielded a repression of expression of −1.25 log_2-fold (Fig. 4). This represents a very important difference between the two normalization strategies obtained using the same input data, and use of an incorrect normalization strategy can lead to entirely incorrect conclusions. In general, normalization with unstable reference indexes (strategies III to V) led to an underestimation of the expression values. As expected, the results obtained with the first two strategies yielded strong correlations of 0.978 for *mnp3* and 0.995 for *lacc10*, which substantially decreased when using indexes of lower quality (see Tables S3 and S4 in the supplemental material).

The five gene expression sets (one for each normalization strategy) were further analyzed using hierarchical clustering combined with a heat map (Fig. 6). The exploratory analysis separated two main clusters corresponding to the *lacc10* and *mnp3* genes, regardless of the normalization strategy. Within each group, strategies I and II always clustered separately from strategies IV and V. The Mann-Whitney test confirmed that there were not significant differences ($P < 0.05$) between normalization with strategy I and normalization with strategy II, regardless of the gene tested. In contrast, normalization with strategies IV and V always led to significantly different results. In the case of strategy III (normalization with a single gene), though *sar1* was proven to be stable, the results were significantly different for the *mnp3* gene. In summary, our results confirm that at least three validated reference genes are needed for obtaining reliable results when normalizing RT-qPCR data.

Expression profiles of *lacc10* and *mnp3*. The transcriptional regulation of the genes *lacc10* and *mnp3* is critically affected by the medium composition (26, 27, 39, 40). To analyze expression changes, we focused on the results obtained by use of the optimal normalization strategy (strategy II). Both genes showed differences in transcription according to the medium composition ($P < 0.05$; see Table S5 in the supplemental material) and growth stage.

The trends in the expression of the two genes were similar within glucose-based medium (SmF) and within the two lignocellulose-based media (s-SSF and SSF), although the induction occurred earlier in *lacc10* than in *mnp3*. The highest level of expression of *lacc10* was found after 3 days of culture in s-SSF medium (ratio = 37.84), and the lowest was found after 7 days of culture in glucose-based SmF medium (ratio = 0.22) (Fig. 5). For *mnp3*, the highest level of expression was also found in s-SSF medium (day 11; ratio = 8.86), and again, the lowest was found in SmF medium (day 7; ratio = 0.79). In SSF medium, *lacc10* had a trend similar to that in s-SSF medium, showing peak expression during the initial stages of growth (day 3; ratio = 11.8), followed by a gradual decrease. In contrast, *mnp3* showed a more constant expression throughout the cycle. Specifically, the *lacc10* expression profile in

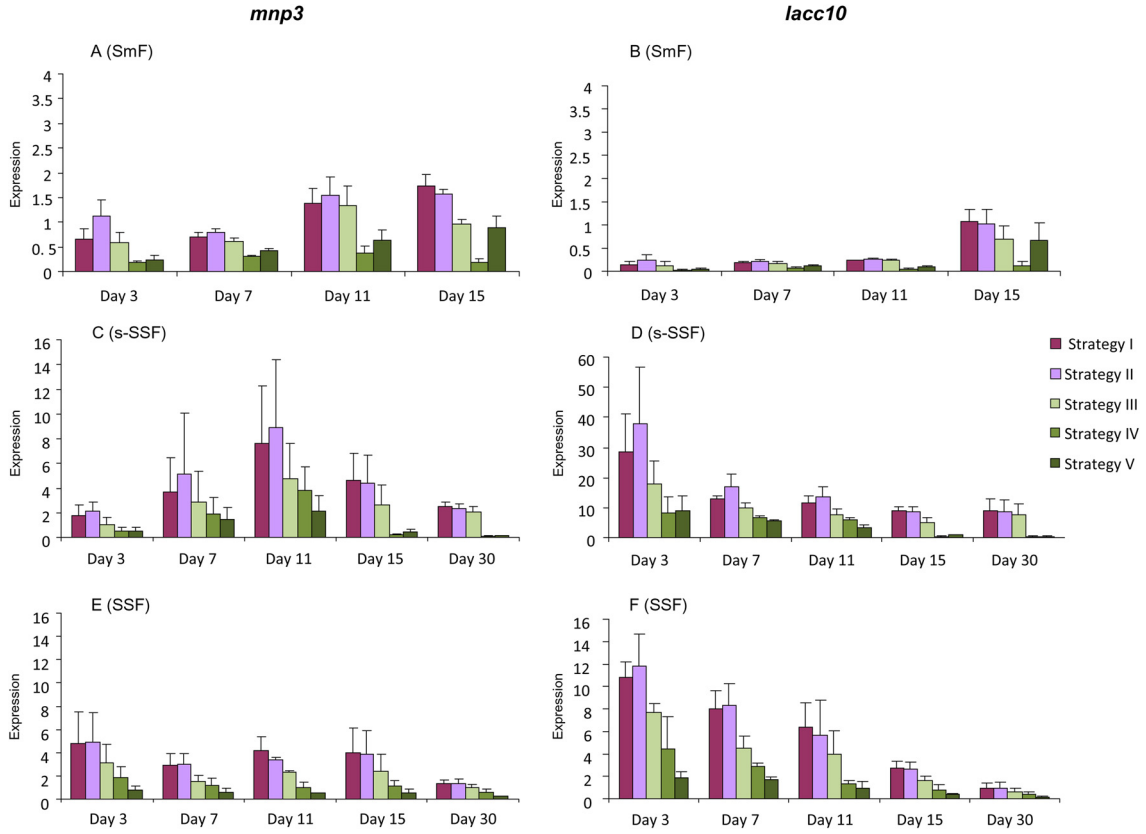

FIG 5 Gene expression ratios (linear) of *mnp3* and *lacc10* in SmF, s-SSF, and SSF media obtained using five different normalization strategies.

SmF medium was correlated with the typical growth curve of *P. ostreatus* in rich media (24), as its expression rose in concordance with the biomass increment and sugar consumption. Nevertheless, the profiles in the s-SSF and SSF media were exactly the opposite, with expression decreasing along the time course. The expression trend for *mnp3* in SmF was similar to that for *lacc10*, while the profiles in s-SSF and SSF media did not suggest a clear correlation with the growth stage.

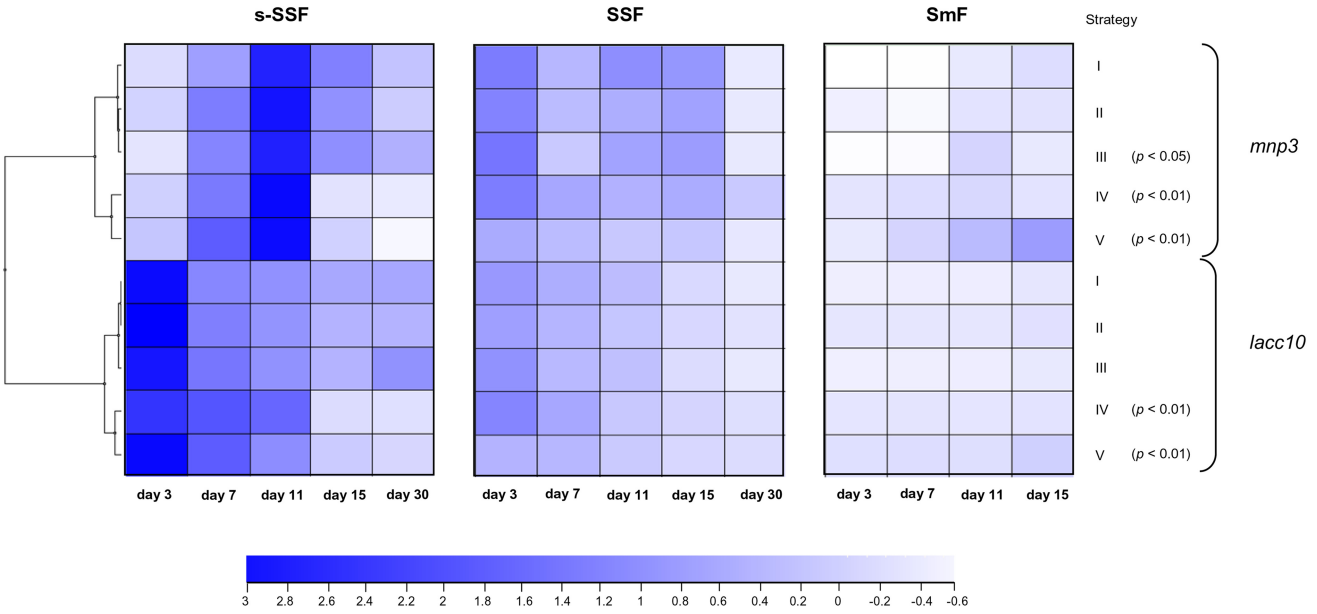

FIG 6 Exploratory analysis of the autoscaled fold-expression ratios of *mnp3* and *lacc10* obtained using five different normalization strategies.

The higher level of expression in the lignocellulose-based s-SSF and SSF media than in the glucose-based SmF medium most likely reflects the requirement of these enzymes in a lignin-rich environment. Laccase production by lignin-degrading fungi in culture media is regulated by aromatic compounds related to lignin (24, 39), metals (41, 42), and nitrogen and carbon sources (43, 44). According to the findings of previous studies, laccase production is highly induced by lignin-related compounds, such as ferulic acid or veratryl alcohol (45), which are present in aqueous wheat straw extracts (24). These compounds can be released into the excess water in s-SSF medium, leading to the induction of laccase transcription. The solid-state condition of the SSF medium would make these compounds less accessible to the hyphae, which could be the reason for the lower level of expression. In the case of SmF medium, the initial repression of *lacc10* likely originated from the glucose concentration in the fresh medium compared with that in the inoculum (which is consumed after 6 days of growth [24]). In other studies, we observed that glucose represses *lacc10* expression while it induces the expression of other laccase genes, such as *lacc6*.

Manganese peroxidases are also known to be regulated by metals, nitrogen, and medium carbon sources (42, 44) and by temperature and pH (27). In this study, we demonstrated that they are overexpressed in lignocellulose-based fermentation in comparison to their level of expression in glucose-based fermentation. The broad spectrum of factors affecting laccase and peroxidase expression makes it difficult to develop an optimal formula. Nevertheless, the s-SSF fermentation model using wheat straw is a cheap and efficient method to obtain amounts of these enzymes larger than the amounts obtained from simple glucose-based media.

The increasing interest in the transcriptional regulation of genes encoding lignin-degrading enzymes frequently involves the use of complex medium, such as wheat straw, wood chips, or other lignocellulose feedstock. The process of mycelium harvesting and subsequent RNA purification is usually difficult to handle in solid fermentation systems based on such media. This often leads to RNA whose quality is poorer than that of RNA from simpler fermentation systems based on soluble carbon sources. As the RNA quality critically affects RT-qPCR assays, the use of stable reference genes is essential for the reliable quantification of expression when comparing samples obtained from different media. In that sense, the availability of an easy and robust protocol with a variety of potential reference genes, like the one described in this paper, will help the scientific community better monitor gene transcription and address experimental findings in a more precise and straightforward way. Moreover, this will be especially relevant when comparing the gene expression profiles of genes in several fermentation systems and in media of different complexities.

Advances in sequencing technologies are increasing the affordability of whole-transcriptome analyses. Nevertheless, while exploratory analyses of transcriptomes using RNA-seq (i.e., for determination of the expression profiles of different developmental stages) are becoming frequent, the need for biological replicates to achieve proper statistical robustness makes confirmatory analyses very expensive. Moreover, the results of differential expression analyses using RNA-seq are influenced by data postprocessing and the statistical test/package used for the analysis (46). In that sense, since the publication of guidelines on the minimum information for publication of quantitative real-time PCR experiments (MIQE guidelines) for RT-qPCR assays in 2009 (17) and their increasing adoption by the scientific community, RT-qPCR has become a very robust and reproducible technology. For this reason, in addition to other important advantages, such as the wide amplification range or the small amount of sample needed, RT-qPCR represents the gold standard of expression analyses. In this context, RT-qPCR and RNA-seq will probably evolve as complementary approaches for studies aimed at answering the biological questions underlying changes in differential expression.

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R.C. conceived of and designed the experiments and analyzed the results, L.L.-V. carried out the qPCR experiments, and A.G.P. revised and edited the manuscript. L.R. led and coordinated the project. The manuscript was written by R.C.

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